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Upregulated ank expression in osteoarthritis can promote both chondrocyte MMP-13 expression and calcification via chondrocyte extracellular PP_i excess

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Summary

Objective: In idiopathic chondrocalcinosis and in osteoarthritis (OA), increased extracellular PP_i ($ecPP_i$) promotes calcification. In chromosome 5p-associated familial chondrocalcinotic degenerative arthropathy, certain mutations in the membrane protein ANK may chronically raise $ecPP_i$ via enhanced PP_i channeling. Therefore, we assessed if dysregulated wild-type ANK expression could contribute to pathogenesis of idiopathic degenerative arthropathy through elevated $ecPP_i$.

Design: Using cells with genetic alterations in expression of ANK and the PP_i-generating nucleotide pyrophosphatase phosphodiestrase (NPP) PC-1, we examined how increased ANK expression elevates ecPPI, testing for codependent effects with PC-1. We also evaluated the effects of ANK expression on chondrocyte growth, matrix synthesis, and MMP-13 expression and we immunohistochemically examined ANK expression *in situ* in human knee OA cartilages.

Results: Using cells expressing defective ANK, as well as PC-1 knockout cells, we demonstrated that ANK required PC-1 (and vice versa) to raise $ecPP_i$ and that the major $ecPP_i$ regulator TGFβ required both ANK and PC-1 to elevate $ecPP_i$. Upregulation of wild-type ANK by transfection in normal chondrocytes not only raised $ecPP_i$ 5-fold to ~100 nM but also directly stimulated matrix calcification and inhibited collagen and sulfated proteoglycans synthesis. In addition, upregulated ANK induced chondrocyte MMP-13, an effect that also was stimulated within 2 h by treatment of chondrocytes with 100 nM PP_i alone. Finally, ANK expression was upregulated *in situ* in human knee OA cartilages.

Conclusion: Elevation of $ecPP_i$ by ANK critically requires the fraction of cellular PP_i generated by PC-1. The upregulation of ANK expression in OA cartilage and the capacity of increased ANK expression to induce MMP-13 and to promote matrix loss suggest that increased ANK expression and $ecPP_i$ exert noxious effects in degenerative arthropathies beyond stimulation of calcification.

Key words: Chondrocalcinosis, CPPD, PC-1, TGFβ, NPP1.

Introduction

A major mechanism by which chondrocytes prevent articular cartilage from calcifying is by maintaining physiologic extracellular levels of the potent basic calcium phosphate (BCP) crystal deposition inhibitor PP_i.[1]. However, substantial increases in cartilage PP_i generation that result in sustained elevation of extracellular PP_i ($ecPP_i$) in the joint occur in association with cartilage aging and with idiopathic, metabolic, and certain familial forms of chondrocalcinosis, as well as in many subjects with OA[1–6]. Over time, this circumstance stimulates pathologic calcification with calcium pyrophosphosphate dihydrate (CPPD) crystals, a common problem that can manifest with substantial intra-articular inflammatory changes and clinical symptoms[1,5,6]. Hydrolysis of excess PP_i also paradoxically promotes cartilage BCP crystal formation in degenerative joint disease via elevated Pi generation[1,3,7].

PP_i is generated both as a by product of numerous biosynthetic reactions and through hydrolysis of ATP and other nucleoside triphosphates (EC 3.6.1.8) by nucleotide pyrophosphatase phosphodiesterase (NPP) ectoenzymes[3,7]. Importantly, intra-articular NPP activity increases concordantly with PP_i generation in a donor age-dependent manner linked to idiopathic chondrocalcinosis in human knees[3,4,6,8]. Three distinct NPP isoenzymes (NPP1-3) are expressed in bone and cartilage, and PC-1 (NPP1) and B10 (NPP3) both catalyze intracellular PP_i generation[3,9,10]. However, B10/NPP3 does not appear to directly promote $ecPP_i$ elevation in skeletal cells[3,9,10]. In contrast, studies using osteoblasts and fibroblasts genetically deficient in the NPP isoenzyme PC-1 have revealed that PC-1-induced PP_i generation is required to support 35–50% of the $ecPP_i$ levels maintained by these cell types[11,12]. Furthermore, upregulation of PC-1 (but not other NPP isoenzymes) has been directly linked with calcification by chondrocytes *in situ* and *in vitro*[3].

The regulatory balance for $ecPP_i$ levels between generation of PP_i and pyrophosphatase-catalyzed PP_i hydrolysis is complemented by cellular channeling of intracellular PP_i ($icPP_i$) to the exterior involving the multiple-pass transmembrane protein ANK[7,13–15]. In this context, transfection of wild type ANK decreases $icPP_i$ and elevates $ecPP_i$ concentrations in normal fibroblasts[13]. But homozygosity for a naturally occurring truncation mutation of the putative C-terminal ANK cytosolic domain in *ank/ank* mice apparently causes 'loss of function' of ANK, with consequent

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elevation of icPP$_i$ and depression of ecPP$_i$[13]. Significantly, *ank/ank* mice develop OA associated with cartilaginous BCP crystal deposition, as well as widespread hyperostosis[13], a phenotype remarkably similar to that of PC-1 deficient mice[12,16].

Two investigative groups recently identified linkages of familial CPPD deposition arthropathy to certain autosomal dominant mutations in the *ANKH* gene for the human homologue of ANK on chromosome 5p[13,17]. Preliminary studies suggested that subtle 'gain of function' of certain ANK mutants might increase chondrocyte 'leakiness' for 'PP$_i$' in 5p familial chondrocalcinosis[13]. Functionally significant ANK mutation appears to be rare in sporadic chondrocalcinosis[13]. But ANK expression, like PC-1 expression[18], is subject to regulation *in vitro* by certain growth factors and cytokines that modulate ecPP$_i$ levels[19]. These include TGFβ, a major promoter of elevated ecPP$_i$[6,9,18]. Furthermore, ANK mRNA levels were increased in cultured chondrocytes from cartilages with OA and chondrocalcinosis relative to normal cartilages when the cells were studied 48 h following extraction[19].

Our objectives in this study were to first ascertain how ANK raises ecPP$_i$, by testing for a mechanism dependent on the fraction of PP$_i$ generation attributable to PC-1. Because PP$_i$ modulates not only calcification but also biosynthetic reactions and expression of osteopontin mRNA and possibly other genes in osteoblasts[20], our second objective was to test for the potential of upregulated ANK to modify chondrocyte function and promote degenerative arthropathy. Our last objective was to assess for upregulated ANK expression *in situ* in OA cartilage.

Materials and methods

REAGENTS

Human recombinant TGFβ 1 and IL-1β were obtained from R&D Systems (Minneapolis, MN). All chemical reagents were obtained from Sigma (St Louis, MO), unless otherwise indicated.

ISOLATION, CULTURE, AND TRANSFECTION OF PRIMARY CALVARIAL OSTEOBLASTS FROM *ANK/ANK* AND PC-1/NPP1 NULL MICE

The *ank/ank* and PC-1/NPP1 null mice colonies employed and methods for breeding, use of congenic control normal mice, and genotype screening were as described in detail[20]. Primary cultures of osteoblasts were isolated from calvariae of 0–3 day-old pups (congenic PC-1 +/+ and PC-1 –/–, congenic ANK/ANK and *ank/ank* mice) by sequential collagenase digestion, as described[20]. An enriched cell population of osteoblastic phenotype obtained from the last five collagenase isolations was pooled and seeded at a density of approximately 4×10^4 cells/cm^2 in α-MEM (Gibco-BRL, Grand Island, NY), containing 10% heat inactivated FCS, glutamine (2 mM), penicillin (50 U/ml) and streptomycin (0.5 mg/ml). Transfection studies in osteoblasts were performed using Lipofectamine Plus (Life Technologies, Grand Island, NY) as described[20], with >40% transfection efficiency verified via control β-galactosidase transfection. We used cDNA expression constructs in pcDNA3.1 for wild-type human PC-1[20] and for murine wild-type ANK and the *ank* mutant ANK (designated as ANK, and MUT *ank*, respectively in the Figures)[13], which were provided by Dr David Kingsley (Stanford University, Palo Alto, CA).

ISOLATION, CULTURE, AND TRANSFECTION OF BOVINE ARTICULAR CHONDROCYTES

Articular chondrocytes from normal bovine knees of mature animals of 30–60 months age (Animal Technologies, Tyler, TX) were obtained by dissection and digestion of the tibial plateau and femoral condyle articular cartilage (using 2 mg/ml of collagenase, incubated at 37°C for 18 h), as previously described[21]. We excluded specimens containing fractures, cartilage fibrillation or cartilage erosion. The typical yield of chondrocytes was ~50 million per normal bovine knee. Primary chondrocytes were cultured in DMEM high glucose supplemented with 10% FCS, 1% glutamine, 100 U/ml Penicillin, 50 µg/ml Streptomycin (Omega Scientific, Tarzana, CA) and maintained at 37°C in the presence of 5% CO2 for 7 days following collagenase digestion, prior to initiation of each experiment. All functional studies of chondrocytes were performed in DMEM high glucose supplemented with 1% FCS, 1% glutamine, 100 U/ml Penicillin, 50 µg/ml Streptomycin (Medium A) unless otherwise stated. For transfection studies, aliquots of primary bovine articular chondrocytes (4×10^5 cells each) were plated in 60 mm dishes, allowed to adhere, and then transfected with the indicated constructs using a described, validated Fugene 6/hyaluronidase method with >40% transfection efficiency verified via control β-galactosidase transfection[21]. Following transfection, 1×10^5 cells/well were transferred to polyHEME coated wells of 96-well plates for studies using non-adherent culture in medium supplemented with 50 µg/ml of ascorbate, as described[21].

ASSESSMENT OF MATRIX CALCIFICATION

To quantify matrix calcification by the primary bovine articular chondrocytes, we used a previously described Alizarin Red S binding assay, which was further validated in each experiment by direct visual observation of Alizarin Red S staining in each plate[3]. In brief, following transfection, bovine chondrocytes (1×10^5 cells/well) were cultured in a 96 well plate coated with polyHEME in 1% FCS, 1% glutamine, 100 U/ml Penicillin, 50 µg/ml Streptomycin, 1 mM sodium phosphate and 50 µg/ml of ascorbate[21]. At 10 days, the dishes were stained with Alizarin Red S and quantified for µmol of bound Alizarin Red S per µg DNA in each well, as described[21].

PP$_i$ AND RELATED ASSAYS

PP$_i$ concentrations were determined by differential adsorption on activated charcoal of UDP-D-[6-3H] glucose (Amersham, Chicago, IL) from the reaction product 6-phospho [6-3H] gluconate[9]. Samples were prepared for analyses of icPP$_i$ and ecPP$_i$, and PP$_i$ concentrations were equalized for the DNA concentration in each well, as described[9]. We determined specific activity of NPP by colorimetric assay using p-nitropheylthymidine monophosphate as the substrate at alkaline pH[9]. Alkaline phosphatase (AP) specific activity also was determined colorimetrically[9]. One Unit of NPP (or AP) was defined as one µmole of substrate hydrolyzed per hour (per µg protein in each sample).

ASSAYS OF COLLAGEN AND PROTEOGLYCANS (PG) SYNTHESIS

PG and collagen synthesis were quantified as described in detail[22]. In brief, to assay PG synthesis, aliquots of 1×10^5 cells were plated in wells of 96 well plates coated with

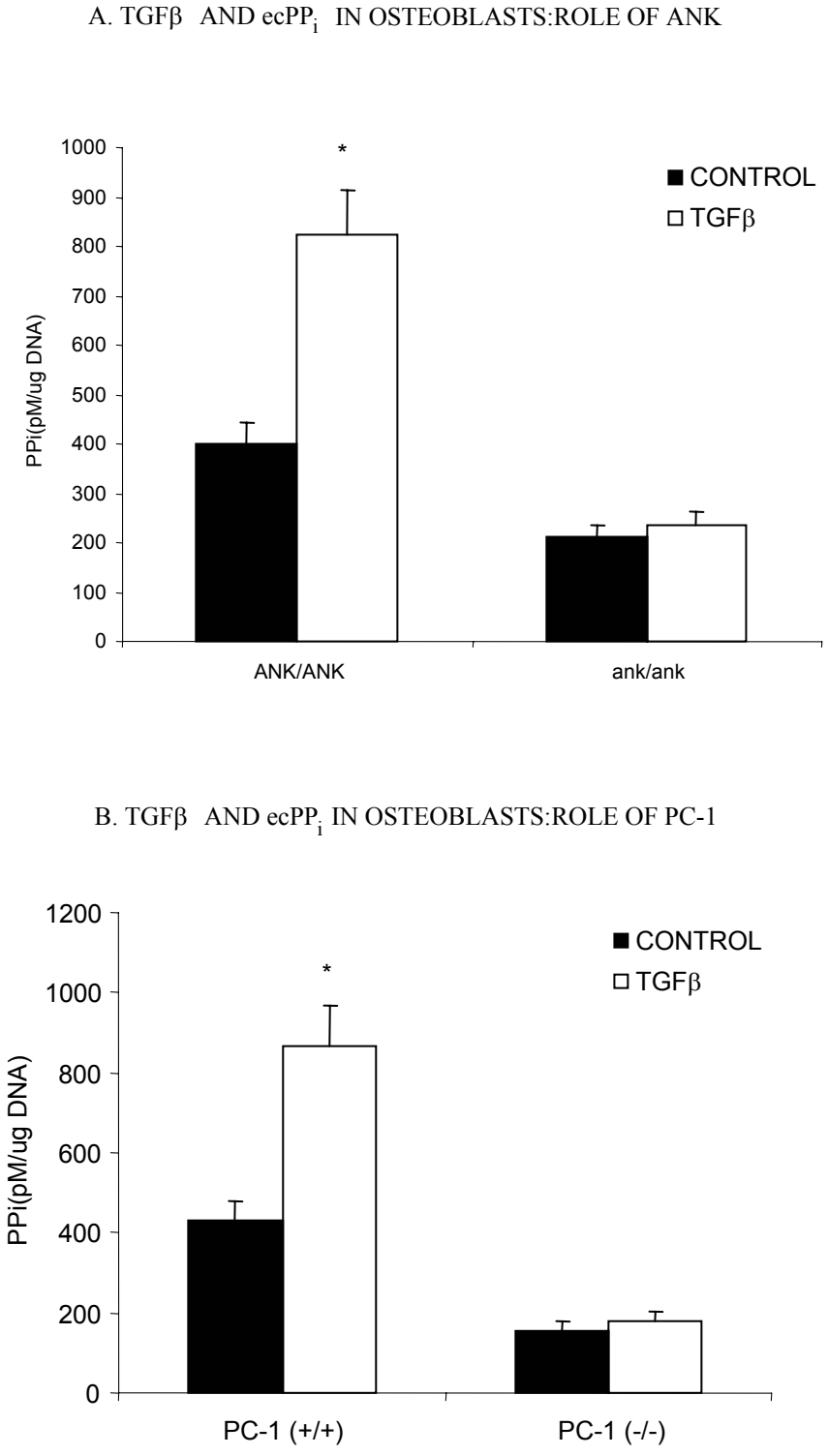


Fig. 1. Requirement for both ANK and PC-1 in TGFβ-induced elevation of ecPP$_i$. Primary calvarial osteoblasts from ANK/ANK and *ank/ank* mice (panel A) or from PC-1 (+/+) and PC-1 (–/–) mice (panel B) were stimulated in monolayer culture (3×10^5 cells/well in a 6-well culture dish) for 48 h with 10 ng/ml of TGFβ in α MEM medium supplemented with 1% FCS. The cell lysates and conditioned media were collected and analyzed for ecPP$_i$ and icPP$_i$, respectively, normalized for cell DNA as described in the Methods. Each assay was run in triplicate and 5 mice of each genotype were studied. *P<0.05.

polyHEME. The cells were cultured for 48 h in the presence of 20 μCi/ml of [^{3}5S] sodium sulfate in the culture medium described above. Conditioned media and cell extracts were then collected and fractionated using a Sephadex G-25M PD-10 column (Amersham Pharmacia, Piscataway, NJ), with elution using 4 M guanidine HCl[22]. We used ^{3}H Proline

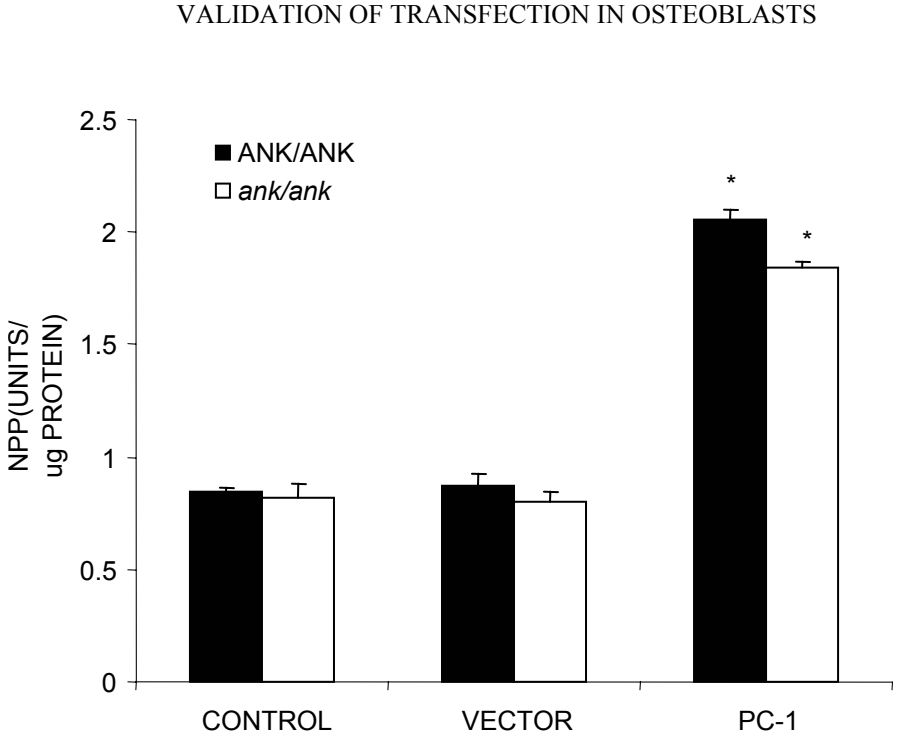


Fig. 2. Validation of functionally efficient transfection of primary osteoblasts with the NPP isoenzyme PC-1. Primary calvarial osteoblasts from ANK/ANK and *ank/ank* mice in monolayer culture (3×10^5 cells/well in a 6-well dish) were transiently transfected with PC-1 cDNA as described in the Methods. After 48 h, the cell lysates were collected and the samples analyzed for NPP activity, with assays run in triplicate and 5 mice of each genotype studied. *$P<0.05$.

incorporation into protein sensitive to 80 U/ml crude collagenase (Worthington Biochemical, Lakewood, NJ) to quantify collagen synthesis[22]. Aliquots of 1×10^5 transfected chondrocytes on polyHEME-coated wells were cultured for 48 h, after which 1 µCi/ml of ^{3}H Proline was added and cells grown for another 24 h. Conditioned media then were collected and precipitated with 15% trichloroacetic acid (TCA), and after repeated washing, the relative amounts of ^{3}H Proline incorporation into collagenase-sensitive protein and total protein were determined. Results for collagen synthesis were expressed as collagenase-sensitive cpm/µg of protein.

ANALYSES OF MMP-13 BY RT-PCR, SDS-PAGE/WESTERN BLOTTING, AND FLUOROGENIC SUBSTRATE ACTIVITY ASSAY

For RT-PCR, total RNA was isolated using TriZOL (Invitrogen, San Diego, CA) and reverse-transcribed and amplified for 35 cycles as described[3]. To assess for MMP-13, forward (5′-CTTCCTCTTCTTCAGCTGGAC-3′) and reverse (5′-ATGTATTCACCCACATCAGG- 3′) primers were used[23], and primers for the ribosomal 'housekeeping gene' L30 were as previously described[21].

SDS-PAGE/Western blotting studies employed previously described methods, in which aliquots (0.01 mg) of protein from each sample were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose[21]. Conditioned media were collected and concentrated with 15% TCA. Anti-MMP-13 (Chemicon, Temecula, CA) antibodies were used at 1:1000 dilution in Western blotting, with immunoreactive products detected using an enhanced chemiluminesence system (Pierce, Rockford, IL), after incubation with horseradish peroxidase-conjugated secondary antibody in blocking buffer for 1 h.

The fluorogenic substrate assay for MMP-13 activity employed aliquots of 1×10^5 primary bovine chondrocytes plated in flat bottom 96 well plates, from which conditioned media were collected. To assay MMP-13 activity, aliquots of 50 µl conditioned media were added to individual wells in a 96-well plate containing 25 µM of the specific MMP-13 fluorogenic substrate MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH2 (Cha=L-cyclohexylalanine; Dpa=3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Nva=L-norvaline) (Calbiochem) in 50 µl of 200 mM NaCl, 50 mM Tris-HCl, 5 mM CaCl2, 20 µm ZnSO4, and 0.05% BRIJ 35, pH 7.5 for 18 h at 37°C. Fluorescence was read at excitation 325 nm, emission 393 nm.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Specimens of normal and degenerative human articular cartilage were taken as full thickness blocks and graded for OA on a scale of I-IV as previously described[8]. For immunohistologic analysis of ANK, frozen sections (5 µm thick) were incubated with 0.5 mg/ml hyaluronidase for 15 min at 37°C, blocked with 10% goat serum for 20 min and incubated for 4 h at 4°C with rabbit anti-ANK 3 polyclonal antibody to ANK[13], also generously provided by Dr. Kingsley. Washed sections were incubated for 1 h at 23°C with biotinylated goat anti-rabbit IgG followed by 1 h incubation with peroxidase-conjugated avidin. Peroxidase activity was detected using the Sigma Fast DAB staining kit, according to manufacturer instructions.

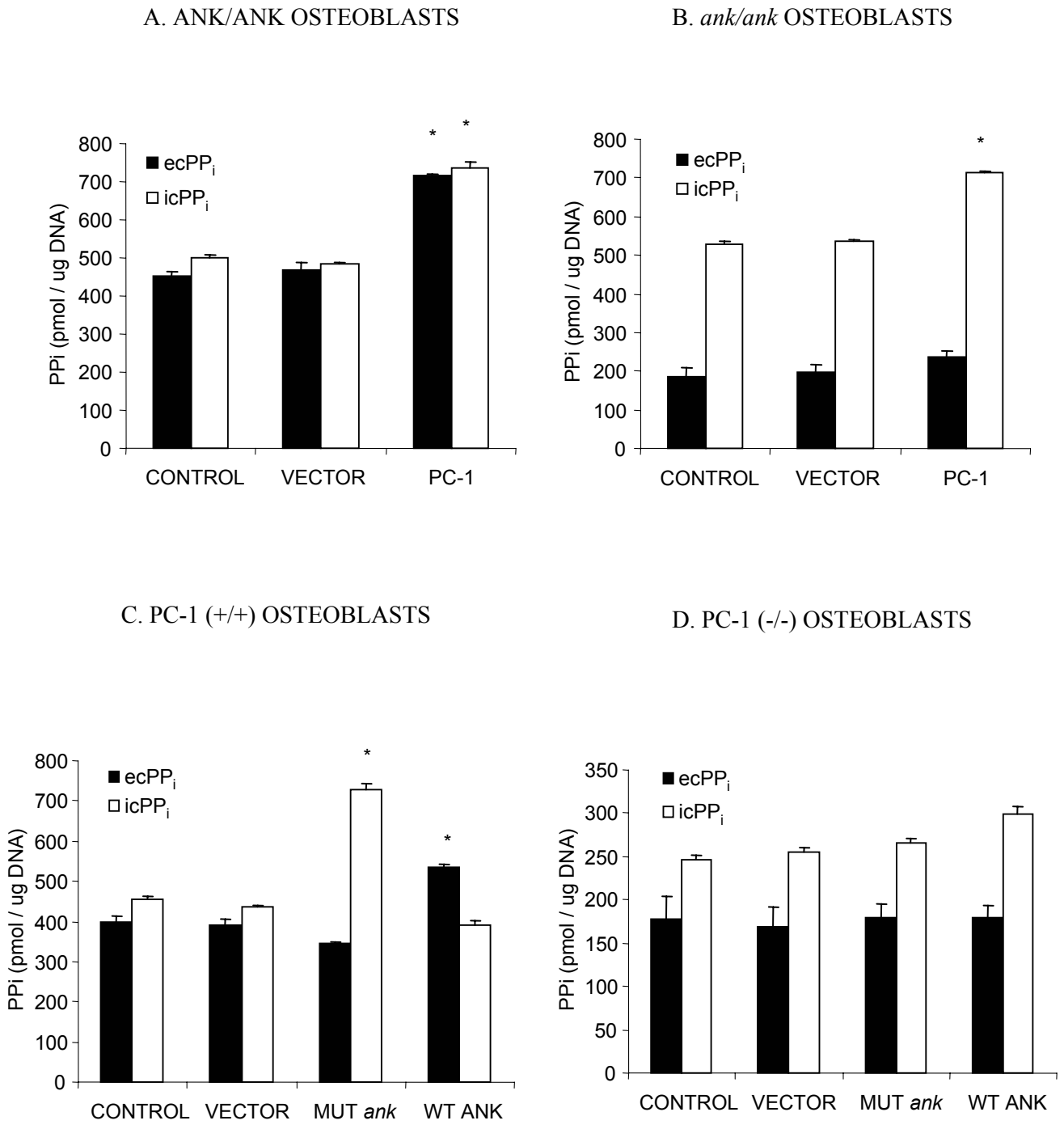


Fig. 3. Co-dependence of ANK and PC-1 in the induction of elevation of ecPP$_i$. Primary calvarial osteoblasts from ANK/ANK (panel A), *ank/ank* mice (panel B), PC-1 (+/+) (panel C), or PC-1 (–/–) mice (panel D) were transfected with PC-1, and wild-type (WT) ANK and mutant (MUT) *ank*, as described above. After 48 h, the conditioned media and cell lysates were collected, heat-inactivated, and analyzed for ecPP$_i$ and icPP$_i$ as described above, with assays run in triplicate. Ten mice of each genotype were studied. *P<0.05 by ANOVA and Student's *t*-test.

STATISTICS

Where indicated, error bars represent SD. Statistical analyses were performed using the Student's *t*-test (paired 2-sample testing for means), and, where indicated, additionally by ANOVA.

Results

ANK AND PC-1/NPP1 CO-ORDINATELY PROMOTE ECPP$_I$ ELEVATION

We examined ANK function *in vitro*, first focusing on how upregulation of ANK modulates ecPP$_i$. Because of the

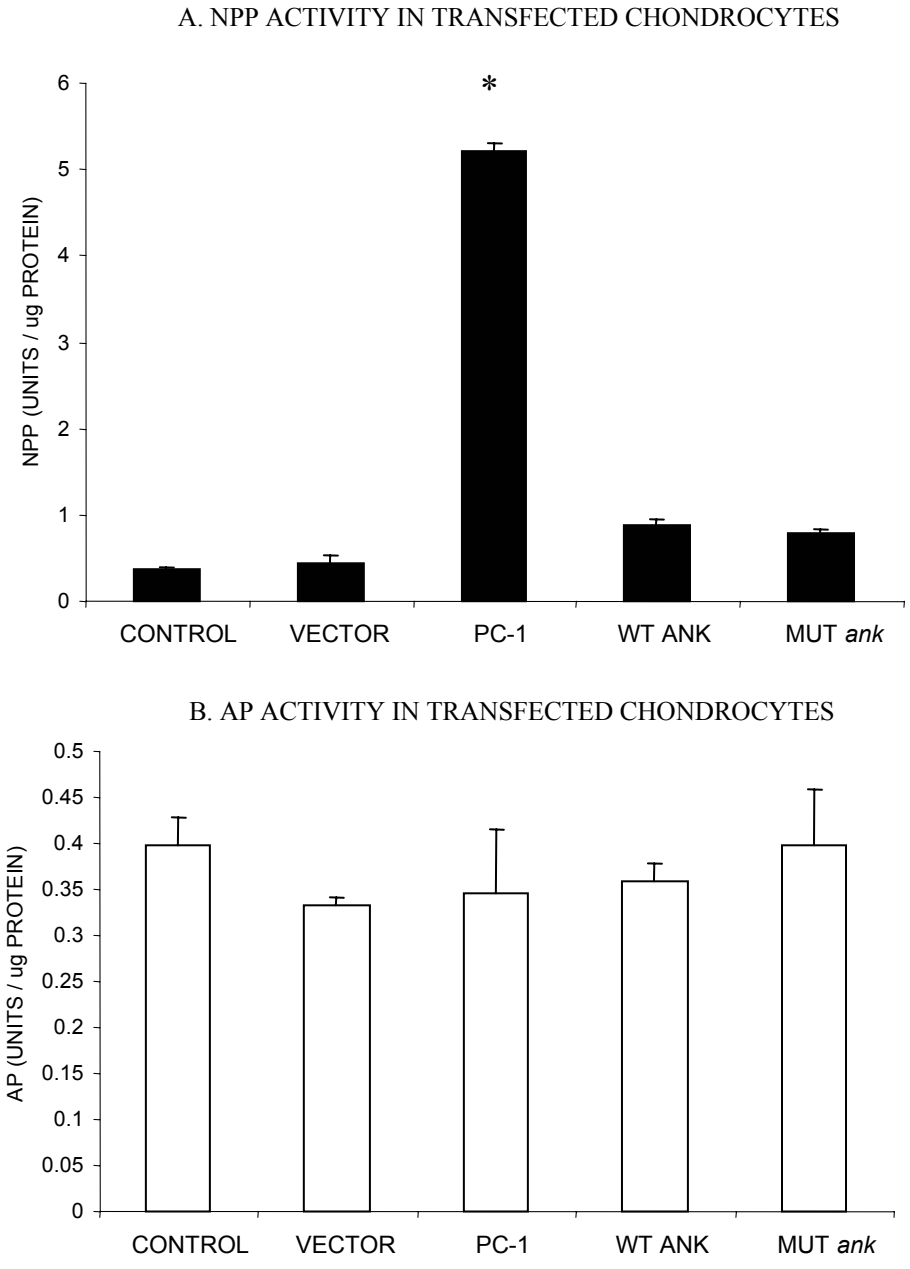
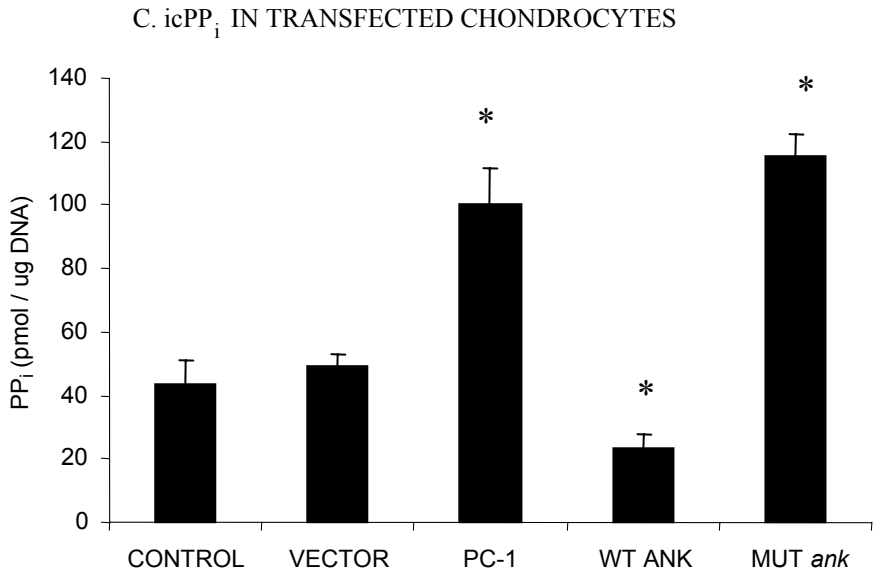


Fig. 4. A and B.

remarkably similar phenotypes of *ank/ank* and PC-1/NPP1-deficient mice[13,16,20], we tested the hypothesis that ANK promotes elevation of $ecPP_i$ in a co-ordinated manner involving the fraction of cellular PP_i generated by PC-1. First, we used PC-1 (–/–) and *ank/ank* cells and their respective congenic wild-type (WT) controls. Because of inherent technical limitations in obtaining adequate numbers of purified mouse chondrocytes for these studies, we examined primary calvarial osteoblasts. The $ecPP_i$ levels were approximately 50% lower in PC-1 (–/–) cells and *ank/ank* cells than in respective congenic controls (Fig. 1).

We demonstrated that TGFβ, a major inducer of $ecPP_i$ elevation in cultured chondrocytes and osteoblasts[4,6,7,9], failed to significantly increase $ecPP_i$ in the absence of either normal ANK or PC-1 expression, in contrast to significantly increased $ecPP_i$ induced by TGFβ in respective congenic WT primary mouse osteoblast controls (Fig. 1). Next, using the same general approach, we assessed whether ANK-induced elevation of $ecPP_i$ required PC-1 and vice versa. In doing so, we employed transient transfection of osteoblasts under conditions previously validated to be efficient[20]. We achieved greater than doubling of NPP enzyme activity in both *ank/ank* and WT (ANK/ANK) cells by transient PC-1 transfection (Fig. 2). Under these conditions, transfection of PC-1 failed to increase $ecPP_i$ in *ank/ank* cells, but did so in ANK/ANK cells (Fig. 3, Panels B and A, respectively). Tranfection of ANK significantly increased $ecPP_i$, whereas transfection of the mutant *ank*, which lacks the ANK C-terminal cytosolic domain[13], significantly increased $icPP_i$ in normal (PC-1+/+) cells (Fig. 3C). However, neither ANK nor *ank* significantly altered $icPP_i$ or $ecPP_i$ in PC-1(–/–) cells (Fig. 3D). Therefore, ANK



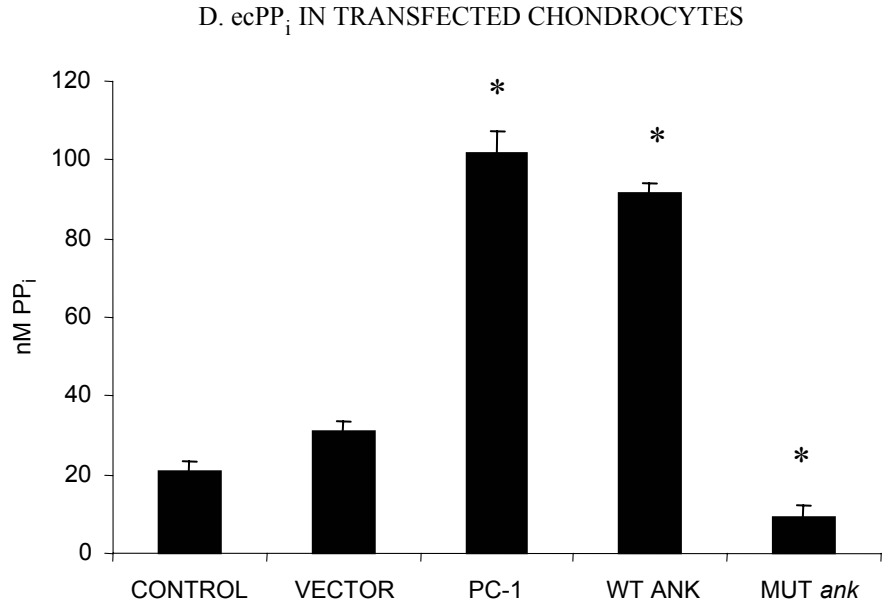


Fig. 4. C and D.

Fig. 4. Effects of transfection of ANK on PP_i levels in primary bovine chondrocytes. Primary bovine chondrocytes were cultured under nonadherent conditions (1×10^5 cells/well in a 96 well plate coated with polyHEME) following transient transfection with cDNA constructs as indicated, using Fugene 6/hyaluronidase as described in the Methods. The cells were incubated for 48 h at 37°C in medium supplemented with 1% FCS and 50 μg/ml of ascorbate, as described in the Methods. Data were pooled from 6 experiments, each performed in triplicate. Panels A, B: The cell lysates were collected in 1.6 mM MgCl2, 0.2 M Tris, pH 8.1, and 1% Triton X-100 and then assayed for NPP and AP specific activity, as described in the Methods. Panel C: The cell lysates were collected and the $icPP_i$ levels were determined and normalized for cellular DNA, as described above. Panel D: The conditioned media were collected and analyzed for $ecPP_i$, with results expressed here as molar concentration of $ecPP_i$. *P<0.05 by ANOVA and Student's t-test.

regulated PP_i levels in a manner coordinated with PP_i-generating activity specifically contributed by PC-1.

FUNCTIONAL EFFECTS IN CHONDROCYTES OF UPREGULATION OF ANK EXPRESSION VIA $ecPP_i$

We next examined the *in vitro* functional consequences of upregulated ANK expression in chondrocytes. We focused on potential effects promoting OA and chondrocalcinosis and the relationship of such ANK-mediated chondrocyte responses to elevation of $ecPP_i$. To do so, we cultured primary normal bovine knee articular chondrocytes under nonadherent conditions using polyHEME-coated surfaces. In addition, we directly upregulated expression of ANK and pertinent controls via a transient transfection approach[21]. To validate our experimental conditions, we

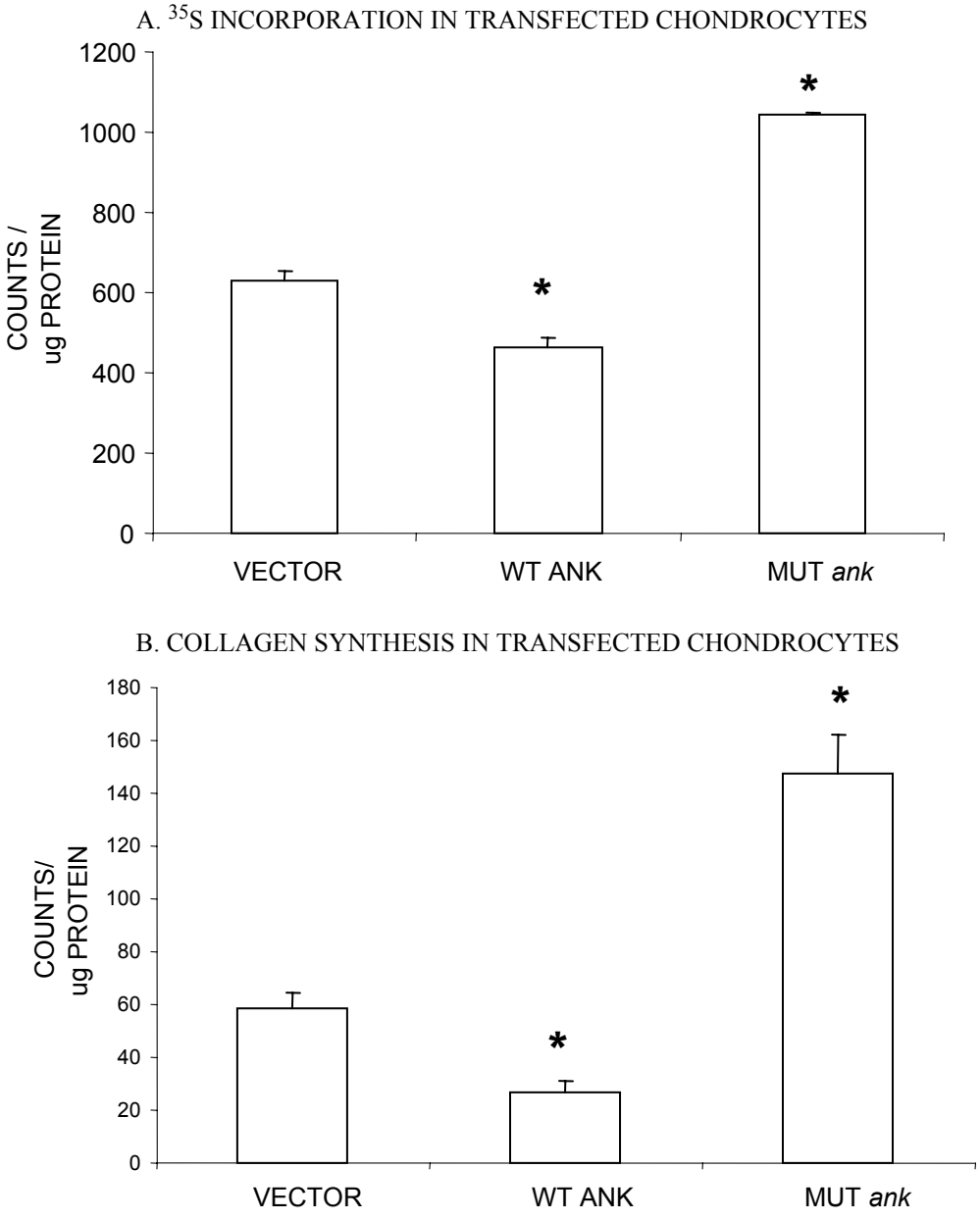


Fig. 5. Transfection of ANK and *ank* alter sulfated PG and collagen synthesis in chondrocytes in opposing directions. For studies of PG synthesis in Panel A, primary bovine chondrocytes, cultured in nonadherent conditions (1×10^5 cells/well in a 96 well plate coated with polyHEME) following transfection, were incubated in triplicate for 48 h at 37°C in 1% stimulation medium containing 20 μCi/ml of [35S] sodium sulfate and 50 μg/ml of ascorbate for 48 h. The conditioned media and cells were collected, extracted and eluted from Sephadex G-25M PD-10 columns. Panel B: Primary bovine chondrocytes, cultured in nonadherent conditions (1×10^5 cells/well in a 96 well plate coated with polyHEME) following transfection, were incubated for 72 h at 37°C in medium supplemented with 1% FCS. During the last 24 hours, 1 μCi/ml of ^{3}H Proline was added to the media. The media were collected and precipitated with 15% TCA and the ratio of ^{3}H incorporated into collagenase sensitive protein relative to total protein used to calculate collagen synthesis, expressed as cpm/μg of protein, as described in the Methods. Experiments were run in replicates of 3, with data compiled from 5 experiments. *P<0.05.

verified that PC-1 transfection, but not transfection of the ANK or the *ank* mutant, markedly elevated NPP specific activity in the primary chondrocytes (Fig. 4A).

Next, we assessed $ecPP_i$ and $icPP_i$ under these conditions, in which transfection of PC-1, ANK, and *ank* did not significantly affect the specific activity of the major chondrocyte PP_i-degrading ecto-enzyme AP (Fig. 4B). We also validated that direct upregulation of ANK significantly decreased $icPP_i$, with the opposite effects stimulated by forced *ank* expression and PC-1 expression in the primary chondrocytes (Fig. 4C). ANK and PC-1 both were confirmed[3,9,13] to induce increased $ecPP_i$ levels. The concentrations of $ecPP_i$ transfected with ANK and PC-1 went up by approximately 5-fold to reach ~100 nM for the chondrocytes (Fig. 4D). Under these conditions, sulfated PG synthesis and collagen synthesis in chondrocytes were suppressed by ANK but augmented by *ank* transfection (Fig. 5A,B). In chondrocytes carried for a substantially

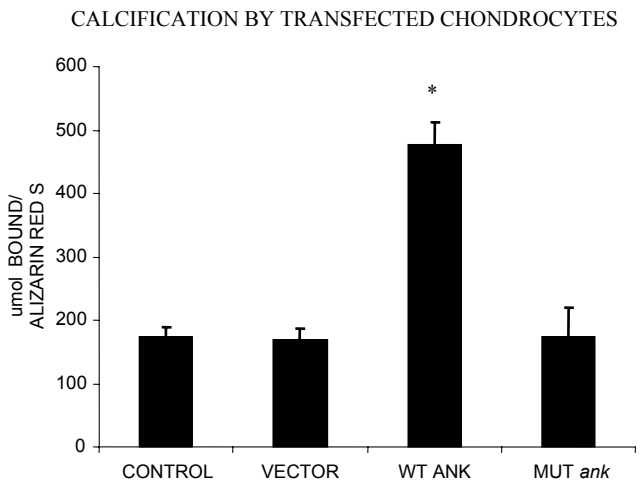


Fig. 6. Transfection of ANK increases matrix calcification in primary bovine chondrocytes. Following transfection, primary bovine chondrocytes were plated (1×10^5 cells/well) in individual wells of 96 well plates previously coated with polyHEME. The cells were cultured in media supplemented with 1% FCS, 50 μg/ml of ascorbate and 1 mM sodium phosphate, as described in the Methods. Calcification was measured at 10 days by binding of insoluble Alizarin Red S, as described in the Methods, with each experiment run in replicates of eight and the data pooled from four separate experiments. *P<0.05.

longer period (10 days), the upregulated expression of ANK induced more than doubling of matrix calcification (Fig. 6). In contrast, transfection of *ank* did not significantly affect matrix calcification within this time frame (Fig. 6).

Because MMP-13 is a central regulator of matrix degradation in OA[24], we next studied and compared the effects of ANK and *ank* transfection in primary chondrocytes on expression and activity of MMP-13. Under conditions where 24 h of positive control IL-1 treatment induced MMP-13 mRNA, ANK (but not *ank* or TGFβ) also induced MMP-13 mRNA expression (Fig. 7A). At 7 days, some full length MMP-13 pro-enzyme was detectable by SDS-PAGE/ Western blotting in the conditioned media of all cells. But detection of a lower molecular weight activation band of MMP-13 was unique to the media of cells treated with IL-1 or transfected with ANK (as opposed to cells transfected with *ank*) (Fig. 7B). As measured by specific fluorogenic substrate assay, conditioned media MMP-13 activity rose, but only modestly so, with IL-1-treatment (Fig. 7). ANK transfection induced relatively marked MMP-13 activity (Fig. 7C).

Given the distinct effects of ANK and *ank* on both $ecPP_i$ and MMP-13 (Fig. 7A), we next tested if increased $ecPP_i$ played a direct role in effects of ANK on MMP-13 (Fig. 8). To do so, we pulsed chondrocytes with exogenous PP_i (10 nM to 10 μM) for 2 h. We observed rapid induction of MMP-13 mRNA in chondrocytes treated with ≥100 nM PP_i (Fig. 8).

UPREGULATED ANK EXPRESSION *IN SITU* IN HUMAN KNEE OA CARTILAGE

In view of the deleterious responses of chondrocytes to upregulated ANK *in vitro*, we next evaluated for upregulated ANK expression in OA cartilage *in situ*. Constitutive ANK expression was detectable in normal human knee

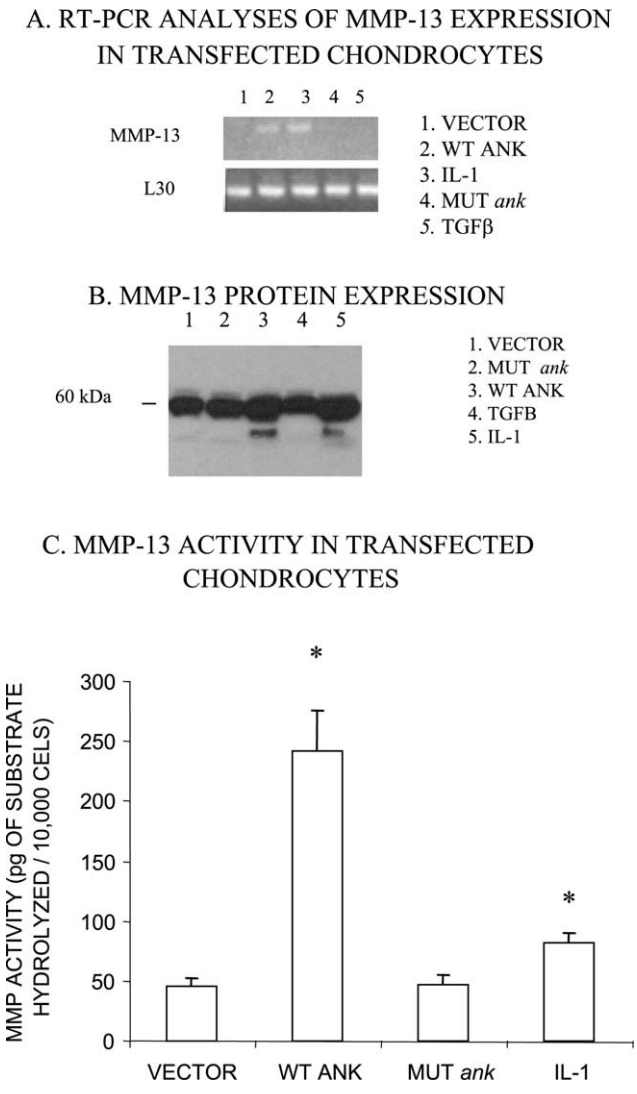


Fig. 7. Upregulated expression of WT ANK induces increased MMP-13 expression and activity. Panel A. Following transfection with vector DNA alone or the indicated cDNA constructs in the same vector, primary bovine chondrocytes were cultured in monolayer (3×10^5 cells/well in a 6 well dish) for 24 h. Where indicated, IL-1 or TGFβ (10 ng/ml) were added to vector control-transfected cells. The total RNA was collected and reversed transcribed as described in the methods. Thirty-five cycles of PCR were performed for MMP-13 and the housekeeping protein L30. Panel B. Following transfection with vector control or the indicated cDNA constructs in the remaining samples, primary bovine chondrocytes were in cultured in monolayer (3×10^5 cells/well in a 6-well dish) for 7 days. Where indicated, IL-1 or TGFβ (10 ng/ml) were added to vector control-transfected cells. The conditioned media were collected, concentrated and aliquots of 0.01 mg were separated by SDS-PAGE and studied for MMP-13 by Western blotting, as described in the Methods. The higher molecular immunoreactive MMP-13 bands represent pro-enzyme and the lower molecular weight immunoreactive bands represent potentially activated enzyme. Panel C. Following transfection and treatments as described in Panel B above, primary bovine chondrocytes were cultured in monolayer (3×10^5 cells/well in a 6 well dish) for 7 days. The conditioned media were collected and analyzed for MMP-13 activity per cell number via assay for cleavage of a fluorogenic substrate specific for MMP-13, with each experiment run in replicates of three and data pooled from 5 separate experiments. *P<0.05.

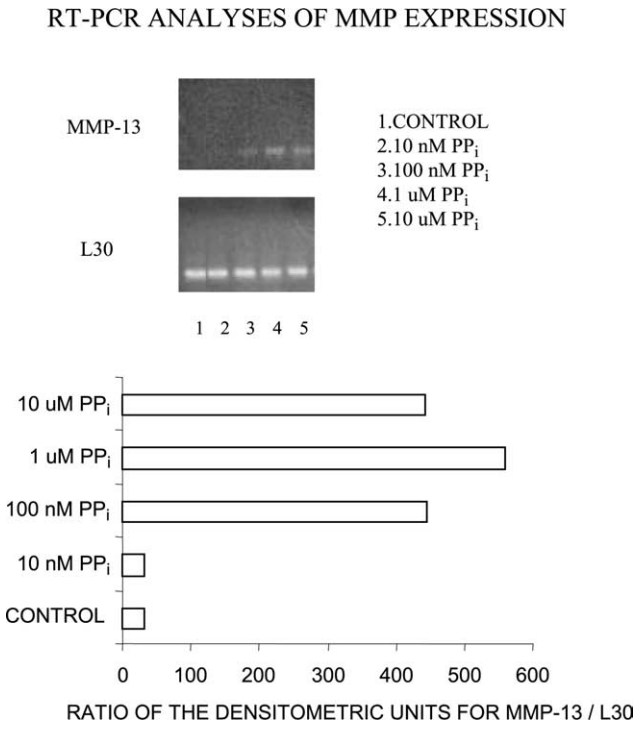


Fig. 8. PP_i excess rapidly induces MMP-13 expression. Primary bovine chondrocytes were in cultured in monolayer (3×10^5 cells/well in a 6 well dish) for 2 h in the presence of the indicated concentrations of PP_i achieved by direct supplementation of the media with sodium PP_i. At 2 h, total RNA was collected and reversed transcribed, as described above, for RT-PCR analyses of MMP-13 and L30. Indicated in the lower part of the Figure are the results of densitometric analyses of the RT-PCR data for the ratio of the MMP-13 mRNA relative to the respective L30 mRNA control.

cartilages at a low level and predominantly in chondrocytes of the superficial zone, with substantially less intense ANK expression detected in the middle and deep zones (Fig. 9A). Detection of robust ANK expression in all zones of knee cartilage was a feature of OA, with Fig. 9B-C demonstrating representative stained sections from subjects with grades II and III cartilage lesions. Therefore, ANK expression was upregulated throughout OA cartilages *in situ*.

Discussion

The basic mechanisms for the strong associations of elevated intra-articular $ecPP_i$ with articular cartilage aging and various degenerative arthropathies[1–6] have been subject to re-evaluation in light of recent findings. Specifically, results of prior studies on cartilage explants or chondrocytes treated with supraphysiologic exogenous ATP, or trypsinized to nonspecifically cleave membrane proteins, were interpreted to indicate that elevated $ecPP_i$ levels in cartilage principally arises from extracellular ATP hydrolysis by NPP ecto-enzyme activity resident on the plasma membrane[25–28]. But this paradigm has subsequently been called into question. In specific, the anion transport inhibitor probenecid at millimolar concentrations not only inhibited TGFβ-induced $ecPP_i$ elevation[29] but also inhibited the capacity of ANK to elevate $ecPP_i$[13]. Altered function of at least one mutant form of ANK, a promoter of intracellular PP_i channeling to the cell exterior, also appeared to increase chondrocyte 'leakiness' for PP_i in autosomal dominant chromosome 5p-linked familial chondrocalcinosis[15].

The results of this study further supported a central role of $icPP_i$ to $ecPP_i$ channeling by ANK in the modulation of $ecPP_i$ by skeletal cells. Specifically, we analyzed how normal ANK functions to elevate $ecPP_i$ using recombinant ANK and primary osteoblasts with genetically mediated alterations in PP_i generation and transport. By transfecting ANK, we determined that ANK elevated $ecPP_i$ in a manner dependent on PC-1 NPP activity. Correspondingly, transfection of the *ank* mutant failed to elevate $icPP_i$ in PC-1 (–/–) cells. In addition, *ank* appeared to exert a 'dominant negative' effect on intracellular to extracellular PP_i movement in normal cells transfected with *ank*.

TGFβ, which becomes upregulated in OA cartilage[30], exerts profound effects on $ecPP_i$ levels in chondrocytes and osteoblasts[20,31]. In this study, both PC-1 and ANK were critical for $ecPP_i$ elevation induced by TGF . Furthermore, TGFβ induces both PC-1[18] and ANK expression[19], and TGFβ stimulates the translocation of NPP activity to the plasma membrane and into plasma membrane-derived matrix vesicles[9,18,32]. The results of this study suggest that the intracellular fraction of PP_i made by PC-1, which accounted for ~50% of $icPP_i$ in primary osteoblasts, is the principal PP_i fraction channeled to the cell exterior by ANK. The results also suggest that the channeling by ANK of the $icPP_i$ fraction generated by PC-1 is subject to upregulation by TGFβ. Because the ecto-enzyme activity of transmembrane full-length PC-1 would be expected to generate PP_i intracellularly only within the lumen of organelles[9], our findings raise compelling questions about how PC-1 and ANK co-ordinate to raise $ecPP_i$. For example, it remains to be determined if ANK modulates transport of the NPP substrate ATP, if ANK and PC-1 co-localize intracellularly, and if ANK can channel PP_i generated by PC-1 in the endoplasmic reticulum or in the Golgi or transport vesicles that deliver PC-1 to the plasma membrane[9,33]. Alternatively, soluble forms of proteolytically released PC-1 are known to be generated[34], and intracellular generation of soluble PC-1 might provide a source of cytosolic $icPP_i$ for plasma membrane ANK to channel to the extracellular space.

The results of this study reinforced the major effects of ANK and PC-1 on PP_i metabolism in chondrocytes. However, ANK nor PC-1 are not the sole determinants for the levels of either $ecPP_i$ or $icPP_i$, due to concurrent effects of factors including substrate availability, certain NPP family isozymes other than PC-1, pyrophosphatases and ATPases, and the effects of various cytokines, growth factors, and matrix proteins[3,6,7,9,10,12,18,21,22,25,26,28,29,31]. Indeed, $icPP_i$ can be substantially reflective of the pools of PP_i in the mitochondrion and endoplasmic reticulum as well as cellular biosynthetic activities[7] and $icPP_i$ appears to fluctutate more than $ecPP_i$ in osteoblasts (Johnson K, *et al*, unpublished observations). As such, cell metabolic effects following on transfection conditions, which included adding DNA and performing lipofection for 6 h in low serum conditions, likely accounted for the nearly equal $icPP_i$ levels in wild type and *ank/ank* primary osteoblasts seen here. In a separate study, we have confirmed elevation of $icPP_i$ and depression of $ecPP_i$ in untransfected *ank/ank* mouse primary osteoblasts[20], analagous to findings in mouse fibroblasts[13].

In this study, we demonstrated upregulated ANK expression *in situ* in human knee OA cartilages. A failure of matrix

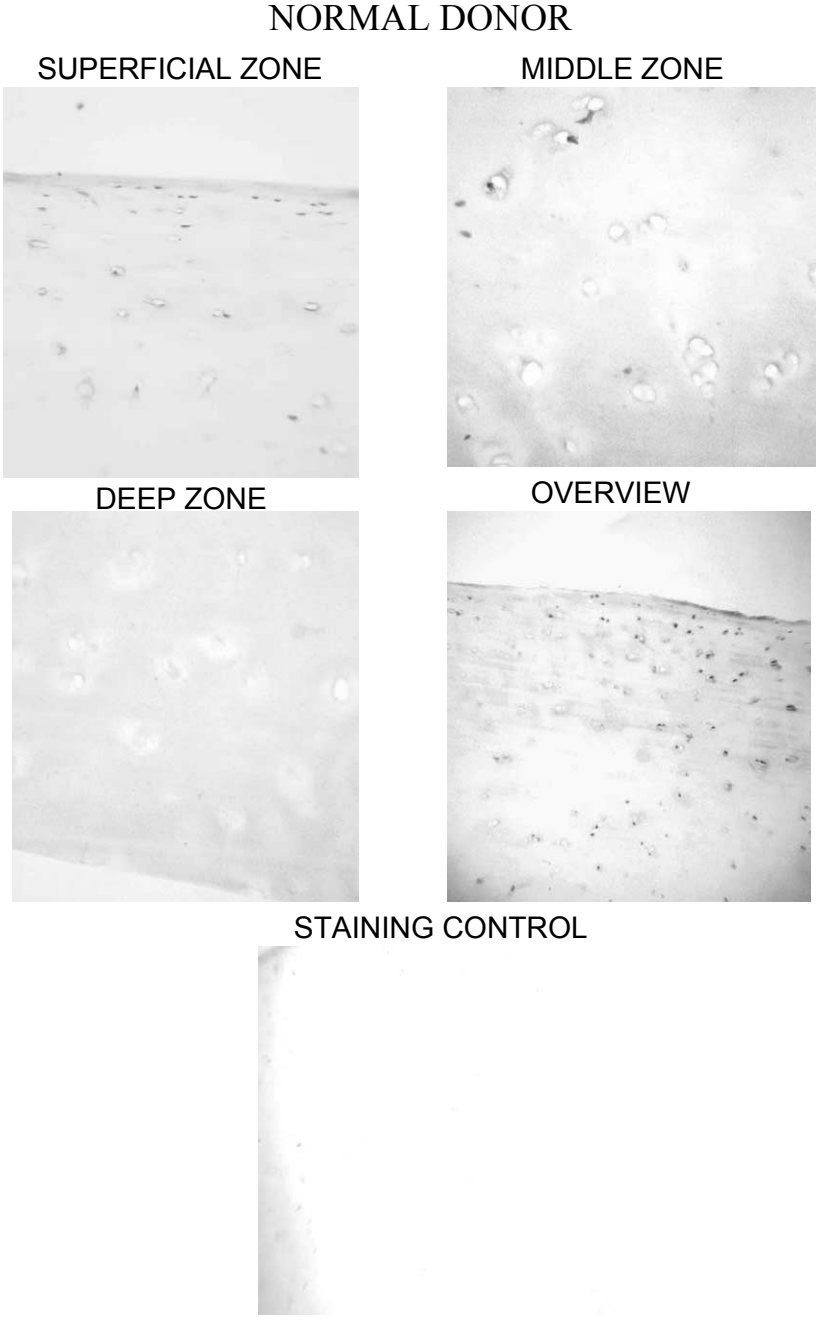


Fig. 9. A.

synthesis to keep up with matrix degradation is a feature of OA[35,36]. Our results suggest that in the setting of OA, upregulated ANK expression may be one of the factors contributing to a failure of cartilage tissue repair. Specifically, we observed that direct upregulation of ANK expression in chondrocytes by transfection *in vitro* not only promoted calcification but also impaired chondrocyte matrix homeostasis. The direct upregulation of ANK induced suppression of both collagen and PG synthesis in chondrocytes. These effects occurred several days in advance of augmented calcification, which requires at least a week to develop in this experimental system[3]. Furthermore, transfection of *ank* induced effects opposite to those of ANK, as *ank* induced increased synthesis of collagen and PG synthesis under the same conditions, without causing a significant change on matrix calcification in chondrocytes. As such, the deleterious effects of upregulated ANK expression on matrix synthesis did not appear to be artifacts from either the transfection of chondrocytes or the capacity of ANK to promote calcification. Indeed, we also have observed significantly increased collagen synthesis in *ank/ank* and PC-1 (–/–) primary mouse osteoblasts relative to their respective normal control congenic osteoblasts (Johnson, K *et al.*, unpublished observations). The precise mechanism by which ANK and *ank* opposingly regulate collagen biosynthesis is not clear, but their contrasting effects on $ecPP_i$ are strong candidates to be centrally involved.

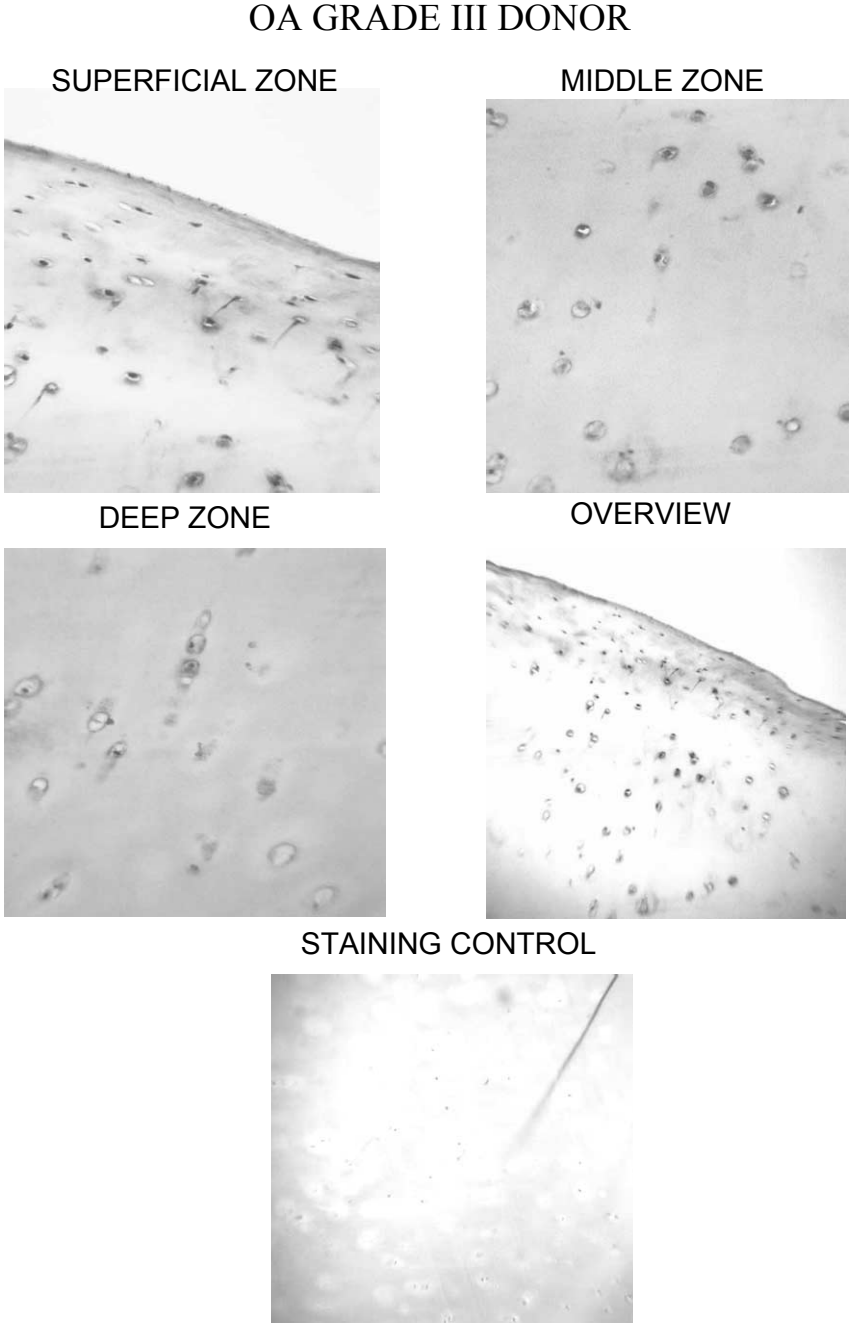


Fig. 9. B.

In this study, we observed that upregulated ANK expression not only impaired matrix synthesis but also induced MMP-13 mRNA expression and, with time, promoted a striking increase in MMP-13 activity. Significantly, the forced upregulation of ANK induced nearly a 5-fold rise in $ecPP_i$, such that chondrocyte $ecPP_i$ levels reached the 100 nM range *in vitro*. We further observed that pulsing of chondrocytes with >100 nM exogenous PP_i induced MMP-13 expression at 2 h of treatment time. Such absolute concentrations of $ecPP_i$ seen with cultured chondrocytes in this study are lower than those reported to be sustained on a chronic basis *in vivo* in chondrocalcinosis joint fluids and to be achieved *in vitro* in a variety of other studies of cartilages or isolated chondrocytes[2,5,6,28,29]. But it should be noted that chondrocytes were examined under conditions of supplementation with only 1% serum and under a limited set of culture conditions in our study. Despite such limitations in extrapolating data to biologic conditions *in vivo*, we believe the novel demonstration of rapid and direct noxious effects for chondrocytes of relative change in $ecPP_i$ levels of ~5-fold is noteworthy. Furthermore, the evidence that upregulation of both ANK and exogenous PP_i rapidly promote MMP-13 expression identify potential effects on chondrocytes of augmented $ecPP_i$ by itself that ultimately promote OA. More extended incubation of chondrocytes with exogenous PP_i was beyond the technical scope of this study, as it requires conditions optimized and validated for generation of sustaining of $ecPP_i$

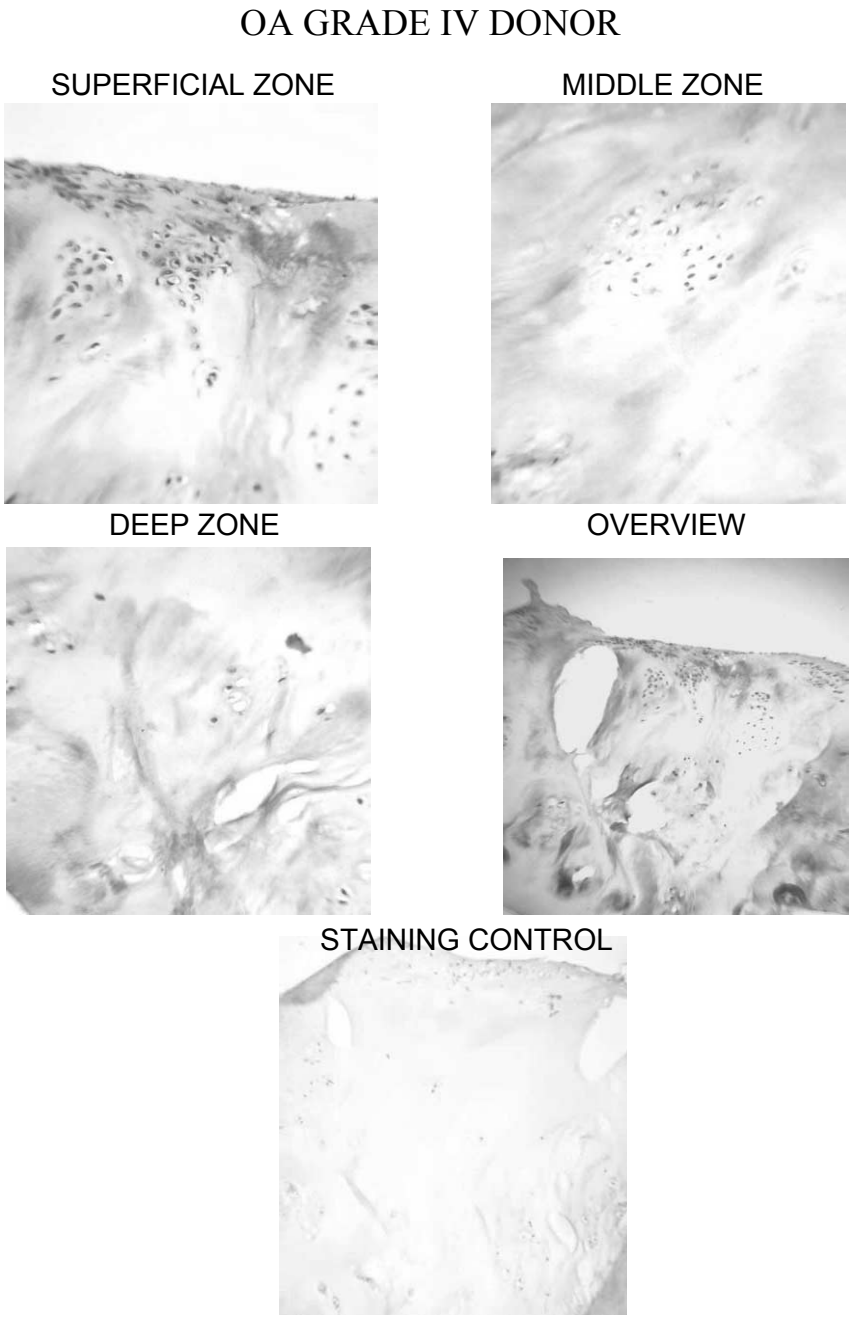


Fig. 9. C.

Fig. 9. Detection of ANK expression *in situ* by immunohistochemistry in normal and OA human articular cartilages. To detect ANK, sections of 5 μm thickness from normal and OA human knee cartilages were stained using polyclonal anti-ANK antibody for 4 h at 4°C followed by incubation with a biotinylated goat anti-rabbit IgG and peroxidase-conjugated avidin, as described in the Methods. For negative staining controls, we used a 1:100 dilution of nonimmune rabbit serum in place of the primary antibody. Panel A shows the analysis of normal cartilages from a normal donor, representative of three studied. Panels B and C are analyses of OA knee cartilages from different donors with grade III and grade IV disease respectively, and the increased intensity of ANK expression (relative to normal cartilages) is representative of five OA knee cartilage donors studied. Magnifications in panels: Superficial Zone, 625X; Middle Zone, 625X; Overview and Negative Staining Control, 150X.

elevation at specific levels by such treatment. We have observed no changes in nitric oxide generation in chondrocytes in which ANK is directly upregulated (Johnson, K, *et al.*, unpublished observations). Thus, the effects of ANK on MMP-13 activity are not attributable to nitric oxide, which is known to promote activation of certain MMPs via S-nitrosylation[37]. It remains to be determined if ecPP$_i$ could directly modulate proteolytic cleavage and activation of the formed MMP-13 pro-enzyme. In this study, we examined articular chondrocytes *in vitro* under culture conditions where formation of the BCP crystal hydroxyapatite is markedly favored[3]. Detailed studies to physically characterize

the crystals induced to deposit by upregulated wild type ANK expression will be of interest using intact normal cartilages and cartilages with matrix changes related to aging and degenerative arthritis. The noxious pro-inflammatory effects of CPPD and BCP crystal deposition in articular cartilages are well-recognized and promote progression of degenerative joint disease[38,39]. The results of this study impart pathophysiologic significance to elevation of cartilage ecPP$_i$ in degenerative arthropathies that goes beyond promotion of pathologic cartilage calcification. As such, the clinical terminology 'pyrophosphate arthropathy' used to describe chronic cartilage degenerative disease associated with CPPD crystal deposition[40] appears to appropriately describe a central feature in pathogenesis. The demonstration of 'gain-of- function' of ANK in idiopathic OA, superimposed on recent findings consistent with 'gain-of- function' of ANK in the degenerative arthropathy familial chromosome 5p chondrocalcinosis[15], indicates that inhibition of ANK-mediated PP$_i$ channeling provides a novel site for potential therapeutic intervention in certain degenerative arthropathies.

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